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Phytoestrogen Project

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Analysis of the T47D-KBluc epithelial breast cancer cell line as a potential model for investigation of phytoestrogen hormones

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Table of Contents

Abstract

Promensil is an over the counter plant based hormonal supplement used to treat menopausal symptoms, and is considered safer than human estrogen treatments. This project evaluates the model cell line, T47D-KBluc, which synthesizes luciferase in response to estrogen receptor binding, which can be used to quantify the binding affinity of each hormone. Using both luciferase assays and PCNA, the cell was examined for its growth and estrogen receptor activity when exposed to Promensil in order to determine that T47D-KBluc is responsive to estrogen like compounds. The findings can be used to support future studies of Promensil and its components in order to develop a better understanding of phytoestrogen drugs.

Introduction

1.1 - Estrogen and Receptors

Estrogens are a family of steroid based hormones that play key regulative and proliferative roles within mammalian organisms. In humans the most common and potent form is 17β-estradiol, but can be found in other forms such as estrone and estriol (Katzenellenbogen et al, 2010). These hormones, which are primarily produced in the ovaries and adrenal glands, influence changes in proliferation and physiology in many areas of the body, most notably in the lumen tissues of the uterus and the tissues of the mammary glands. However, they also interact with cells of the liver and pituitary gland (Hewitt & Korach, 2002).

Estrogens facilitate these changes by binding to specific estrogen receptors (ERs), embedded within the nuclear membrane of target cells. ERs are members of a larger family known as nuclear receptors. These receptors are ligand activated transcription factors that possess highly conserved binding domains, enabling them to bind specific ligands and DNA segments. The ligand binding domain (LBD) is comprised of 12 transmembrane proteins that play a pivotal role in determining the type of ligand that binds to the receptor. In its non - ligand bound form, or Apo LBD, the 12 regions form an anti-parallel α –

helical sandwich. Once bound by a specific receptor, the domain undergoes a conformational change. Several of the transmembrane proteins will adjust their position to form a stable “pocket” for the ligand. In this form, the receptor becomes activated and prepares to bind specific promoter segments.

Before binding to a specific DNA segment, the newly formed ligand + receptor complex will dimerize with other receptors via dimerization loops. These loops consist of zinc finger residues (Cys-476 and Cys482) that allow the receptors to bind together and form stable homodimers. These newly formed complexes possess DNA binding domains that bind and anchor it on specific response regions of the DNA and facilitate gene expression and overall change within the cell. Antagonists are ligands that can bind to nuclear receptors and cause conformational change as well. However, these ligands bind to receptors and produce complexes that are slightly altered. In some cases this altered conformational change disrupts the DNA binding capabilities of receptor and halt gene expression. In the case of ERs, tamoxifen acts as an antagonist, especially within cells of the breast. This hormone binds to the ER, blocking estrogen hormones from binding and prevents them from stimulating cell proliferation (Tropp, 2005). Figure 1 shows both domains within an estrogen receptor.

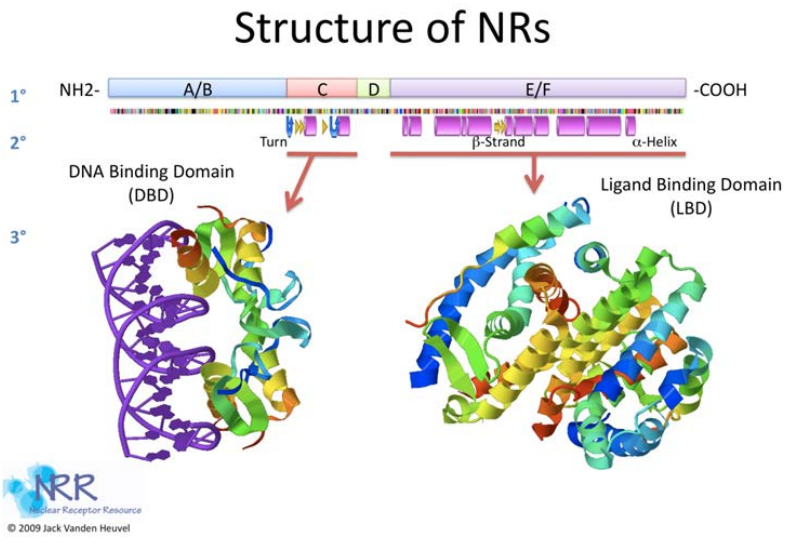

Figure 1: Ligand binding and DNA binding Domains (bio.cmu.edu, 2013)

In human cells, estrogen hormones will interact with two of the main isoforms of the ER, α or β. These ERs share similar overall functions and estrogen affinity. However, they have distinct differences that set them apart. They are distributed uneven throughout the body. ERα have been known to be prevalent in growing cells of the uterus while ERβ exists heavily within areas such as the prostate, ovaries and salivary glands (Hewitt & Korach, 2002). Figure 2, below depicts other areas in the body where these receptors are found.

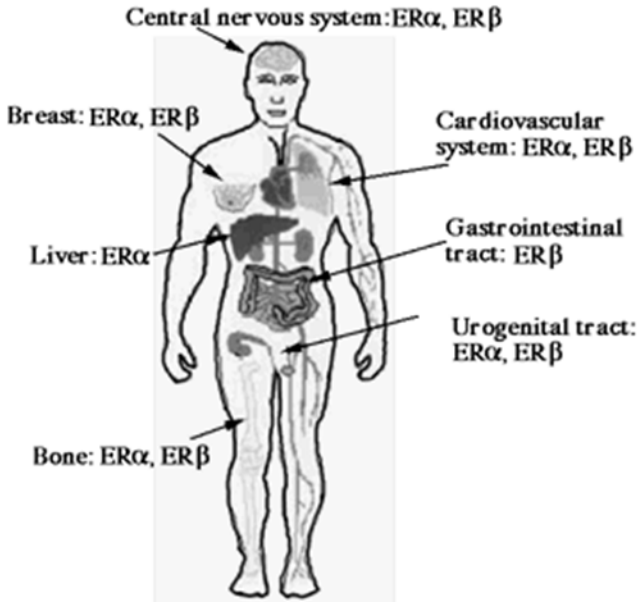

Figure 2: Areas where Er alpha and beta receptors are found (NR Resoource.org, 2013)

They also have slightly different binding regions, causing them to bind differently toward certain ligands. ERα is 30 times more sensitive to genistein, a phytoestrogen that has estrogenic characteristics, compared to its β counterpart. These differences in binding regions also induce results. ERα has been known to promote cell proliferation in areas of the breast and uterus when activated by the 17β - estradiol ligand. However, ERβ has been shown to have anti-proliferative characteristics in these same areas when α receptor is knocked out. Because of these differences, the two receptors are independent of one another.

To activate these receptors estrogenic ligands enter the cell through its plasma membrane and bind to one of the ER isoforms in the nuclear membrane. Once bound by a specific ligand, the ER will

dimerize with another receptor to form a homodimer complex. This complex will bind to an estrogen responsive element (ERE), a 13 base pair repeat sequence. This binding event stimulates the recruitment of other transcription factors and enhancers and stimulates the overall transcription of genes downstream (Katzenellebogen et. al, 2000). ERs possess two domains, C and E, that allow them to effectively bind hormonal ligands and the EREs found in the DNA (Levin, 2005).

1.2 - Hormonal Replacement Therapy

As women age, estrogen levels begins to dissipate as reproductive capabilities fade and they enter menopause. In some women the menopausal stage can be an uncomfortable one, filled with various symptoms including hot flashes and atrophy of the uterus. Women, who are breast cancer survivors, may experience a more extreme form of these symptoms sooner (depending on when the cancer was diagnosed) (Seifert & Kubist, 1999). Female cancer patients often take antagonistic hormones, like tamoxifen, to block estrogen receptors and prevent the possibility of cancerous cell proliferation. Though, this treatment is effective at halting cancerous growth especially within cells of the mammary glands, it still leaves these survivors with reduced estrogen levels (Seifert & Kubist, 1999).

In the past, women with lower level of estrogen have turned to taking estrogen supplement as a therapy for dealing with these adverse effects. Estrogen can provide relief to these symptoms but does place these women at risk to redeveloping cancer (Sakamoto et. al, 2010). Previous studies have shown that the binding of estradiol (E2) to ER causes an up-regulation and increased sensitivity to cyclin D1, a protein that is highly prevalent in cells progressing through the cell cycle. The hormone's binding can also induce anti-apoptotic effects on the cells of the breast. These can lead to unmitigated cell growth and an early sign of cancer within women of this age bracket (Sakamoto et. al, 2010). Because of this potential threat, alternative hormones have been examined in order to find a replacement. Progesterone, coupled with estrogen supplements has produced positive effects by reducing some of the symptoms of menopause. But its risk of stimulating dormant cancer cells in cancer survivors eliminated it as a possible treatment option. Now over the counter phytoestrogen supplements are currently being used to counter act

the reduced estrogen levels and uncomfortable menopausal symptoms that women face as they age (Warren, Halpert, 2004).

1.3 - Phytoestrogens

Numerous phytoestrogens have been investigated to find a safer alternative for hormone replacement therapy. At their core, phytoestrogens consist of a group of plant based hormones possessing an affinity for the ER in estrogen targeting cells (Hopert et. al, 2008). Examples of phytoestrogens being investigated include isoflavonoids, genistein, daidzein, coumestrol and puerarin (Cherdshewasart, Panrainsaen, Picha; 2007).These hormones have attracted attention in recent years for numerous reasons, the first being that they are completely natural product; second, it is believed that some of these hormones can induce estrogen like activity without supporting unmitigated cellular growth. Various studies have tried to analyze the properties of these hormones. A study conducted in 2007 by Doctors Wichai Cherdshewasart Rattana Panriansaen and Porntipa Picha observed the anti-proliferative effects of a plant species known as Pueraria Mirifica (P. Mirifica). In regions of Thailand, this plant has been used and consumed as an herbal remedy for women entering menopause. During the study, this plant was purified via an HPLC revealing several of the phytoestrogens mentions above, suggesting that the plant was phytoestrogen rich. To observe the effects of these phytoestrogens, the plants were broken into powdered form and dissolved in 0.7mL of distilled water. Concentrations of 10, 100 and 1000 mg/kg were fed daily to rats with breast cancer. The results displayed showed rats that received the highest concentrations of the plant powder, produced tumors of a lesser size and diameter than those of the control. Tumor cells treated with the P.Mirifica, possessed lower levels of the estrogen receptor, perhaps explaining the reduction in tumor size (Cherdshewasrt, Panriansaen, Picha, 2007). Though some of the results have been promising, uncertainties still remain. What pathway do these phytoestrogens work through? Does it simply operate through the estrogen receptor or are other receptors involved? Further research is needed to uncover more about these hormones.

1.4 - Promensil

Red clover is the main ingredient in the popular supplement, Promensil, which boasts four main phytoestrogen hormones: daidzin, genistin, formononetins, biochanin A (Setchell et al, 2001). Each of these components could be eaten in a diet of beans and vegetables, but the direct action these components have in concert on human cells is somewhat unknown. Population studies have shown that cancerous diseases of the breast and prostate are lower in the Eastern region of the world, where plant based foods are an essential and significant part of the diet. It has been hypothesized that certain phytoestrogens, such as daidzin and genstein found in soy beans could play a role in this (Lof, Weiderpass, 2006).

1.5 - T47D KBluc

T47D-KBluc has been used in the past to analyze the estrogenic and antiestrogenic properties of certain hormones. The cell line was created using T47D, an adherent breast cancer cell line, commonly used in cancer research. T47D was transfected with triplet estrogen responsive elements (ERE) and a luciferase reporter gene. These components allow the cell to transcribe luciferase in response to certain compounds, human and foreign, acting through the estrogen receptor. As ligand forms a union with multiple estrogen receptors (ER), these receptors will dimerize and bind to corresponding coactivators in the area. These components will then interact with the EREs of the luciferase reporter construct and activate the translation of the luciferase gene further downstream. Luciferin can be added to the cells, causing a reaction with luciferase and producing light relative to the estrogenic activity of the chemical under scrutiny. Quantifying this light can characterize the estrogenic activity of a compound (Wilson et al, 2004).

1.6 – Our aim

In this study, the aim was to determine whether or not the T47D kBluc cell line could act as a model candidate for further investigation of the phytoestrogen mode of action. Luciferase assays were conducted on the cell line to determine the luciferase reporter sensitivity toward phytoestrogen, in

comparison to estrogen. Prior to conducting these assays, we expected the estradiol to cause an increase in luminescence from the cell line. The estradiol would increase its interactions with estrogen receptors, resulting in the overall increase in translation of the luciferase enzyme. We believed that cells exposed to Promensil supplements would display a decreased luminescence in comparison to estradiol. In these cells, interactions with estrogen receptors are reduced resulting in lower amounts of luciferase and luminescence capabilities. Our control, which was cells exposed to ethanol, was predicted to a produce baseline amount of luminescence.

Immunoblots, assaying for the presence of PCNA, were also conducted. PCNA or Proliferative Cell Nuclear Antigen is a known factor in the cell cycle and the proliferation of cells. This protein plays a key role in DNA replication by interacting with the DNA polymerase of the leading strand and increasing its transcriptional efficiency (http://www.uniprot.org/uniprot/P12004). Before conducting the blot, we anticipated that the PCNA signal would be weaker in cells exposed to the Promensil supplements due to the presence of anti-proliferative phytoestrogens (Le Bail et al, 1999). We also believed that cell samples exposed to estrogen would sharply increase the PCNA signal when compared to the control because of its known proliferative properties (Wilson et al, 2004).

Methodology

2.1 Cell Culture

T47D-KBluc cells were cultured in DMEM + 10% FBS + 1% Pen/strep on both T25 and T75 flasks, incubated at 37° C %5 CO_2. Cells were grown for ~96 hours or until high confluence was reached. Flasks were then trypsinized and cultured in either DMEM + 10% FBS (Dextran charcoal stripped) + 1%pen/strep without phenol red or the original media mentioned above. Before use in experimental procedures, cells grew in phenol red free medium for ~96 hours before being plated on either 12 well plates at 100,000 cells per well, or 96 well plates at 10,000 cells per well.

2.2 Hormone Dosing

Soluble Promensil and estradiol concentrations were created using serial dilution techniques. A stock of estradiol at 10^{-7} molar was created using powdered estradiol and ethanol. Tenfold serial dilutions made a range of 10^{-8} to 10^{-13} molar estradiol. To create a soluble Promensil solution, three tablets of Promensil were crushed and refluxed in 100 ml of methanol for 1 hour at ~65° C. Tenfold serial dilutions using ethanol were performed on the refluxed pills to create a range from 10^{-1} to 10^{-4} dilutions of the original. This range of dilutions was created to capture the doses at which human cells would be exposed to in a single day of taking the supplement in accordance to the consumer suggested dosing. The exact concentration of hormones in the Promensil extract was unknown but was considered less than 10^{-7} molar because the extract remained homogenous when refrigerated, whereas 10^{-7} molar estradiol formed crystals.

2.3 PCNA

Cells were grown on 12 well plates at 100,000 cells per well in ~1ml of medium for 24 hours before exposure to hormones. 50ul of ethanol, estradiol solutions 10^{-8} to 10^{-13} and Promensil 10^{-1} to 10^{-4} solutions were added to the wells and incubated for ~24 hours. Media and sample solution was aspirated away to dry the cells before placing the plates in the -80° C freezer to lyse the cells. The contents of the wells were collected by scraping the wells in 200ul of PBS. The protein content of the wells was determined using a Bradford assay. The absorbance was read at 580nm to determine the protein concentration of the samples. Using the results of the Bradford assay the samples were diluted in PBS to normalize the concentration of each sample equal to the lowest concentration of protein. Samples were denatured by boiling for 5 minutes in loading buffer containing SDS, beta mercaptoethanol before being run on a Bio-Rad Mini-Protean TGX 4–20% precast polyacrylamide gel . Following elctrophoretic separation, the proteins were transferred onto a polvinylpyrrolidine membrane using a semi dry blotter to be treated for PCNA detection. The membrane was first exposed to blocking buffer, ~5% non-fat dry milk, for 30 minutes. After rinsing in TBS, 10nM Tris-Cl+150nMNaCl pH7.4, the membrane was

exposed to 0.02% mouse monoclonal Anti-PCNA antibody in TBS+0.1% Tween for enough time to bind to the PCNA bands, ~1 hour at room temperature. The membrane was then exposed to a secondary antibody, Santa Cruz Biotech alkaline phosphatase conjugated gGoat anti-mouse IgG. After rinsing in TBS and TBS with 0.1% Tween-20, the membrane was finally exposed to Sigma Fast BCIP/NBT to visualize the bands.

2.4 Luciferase assay

Cells were grown on 96 well plated at 1,000, 10,000, and 20,000 cells per well in 100ul of phenol red free medium for ~24 hours. 2ul of ethanol, estradiol solution or Promensil solution were added to each well, and the cells were incubated for ~24 hours. 100ul of Promega Steady-Glow® luciferin solution was added to each well to lyse the cells and begin the luciferase reaction. The wells were covered with tin foil to reduce exposure to external light and the reaction was given ~15 minutes to take place. A Perkins Elmer LS55 fluorescence spectrophotometer with a 96 well plate reader was used to measure the well luminescence. Both specific wavelength scans at 560 nm and full spectrum scans of the wells were performed to measure the light output of the luciferase assays, where in every case, the excitation lamp was manually turned off to prevent external excitation of the assay.

Results

3.1 PCNA Blot

Ten samples from the T47D-KBluc cell line were assayed for PCNA production after 24 hours of exposure to varying concentrations of estradiol and the promensil supplement. After conducting a preliminary blot to determine that 100,000 cells produced the optimal PCNA signal, in figure 3, ten samples were placed in electrophoresis wells and assayed for PCNA by immunoblotting as detailed in methods.

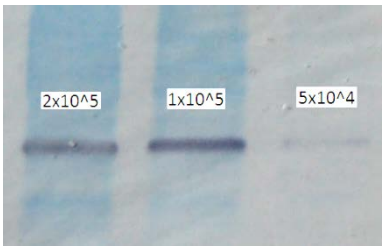

Figure 3. Preliminary PCNA Blot containing with 50,000, 100,000 and 200,000 cell samples

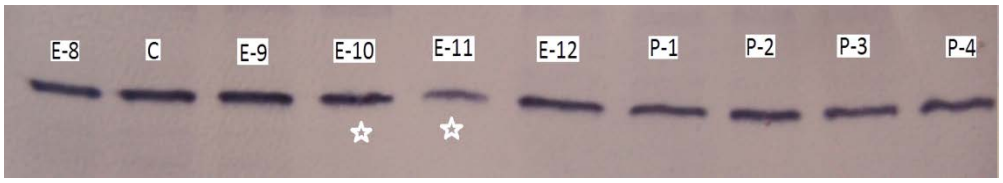

Figure 4. Experimental PCNA Blot containing cell samples dosed with Estradiol and Promensil at concentrations indicated above each band.

Lanes 1, 3, 4 and 5 contain varying concentrations of estradiol. Lanes 6 through 10 contained varying concentrations of promensil. Stars found under lanes 4 and 5 indicate loading errors and should be disregarded.

As seen in Figure 4, there was no discernible visual difference in the signal strength between the control and cells at any level of estrogen treatment. Cells exposed to this hormone produced an almost identical signal to cells exposed to just ethanol, our control. However, lanes containing cells treated with Promensil supplements (lanes 6-10) produced a reduced PCNA signal, supporting the notion that a decrease in cellular proliferation occurred. However there is no visual correlation between the signal strength and the dose of Promensil added.

3.2 Luciferase Assay

Luciferase assays were intended to quantify luminescence emitted from T47D cells following dosing with estradiol and Promensil. Two sets of 12 samples containing 10,000 and 20,000 cells were plated onto a 96 well plate. One set of cells was exposed with ethanol to act as a control while the remaining samples were exposed to the test hormones. Luminescence from this plate was analyzed with a Fluorescence Spectrophotometer and interpreted with the FL Winlab software. The data from the assay proved to be inconclusive. The control samples produced almost identical luminescence values when

compared to the test samples. When the data was displayed on graphs, the observed luminescence covered almost the entire white light spectrum, as opposed to one particular peak close to the predicted 560nm. The assay was additionally performed multiple times with 1,000 and 100,000 cells were per well to determine if the cell concentration could affect the assay. When the cell concentrations were changed, similar results were received from the assay.

Discussion

The PCNA blot performed on cells exposed to both estradiol and Promensil indicated a reduced signaling in lanes containing Promensil, inferring that the Promensil reduced the rate of growth in T47D-kBluc. This data is consistent with what is predicted, though the dosage concentration did not appear to have a noticeable effect on the growth rate, where the signal in lanes with cells exposed to Promensil 10^{-1} and 10^{-3} dilutions appear equivalent. No significant response between dosages could mean that the cells were saturated by the doses, indicating that the lowest dilution induced the same interaction with the cells as the highest. This data implies that a larger dilution pool of the Promensil extract should be examined. In each case, the Promensil reduced PCNA levels, indicating that T47D-kBluc responded to Promensil by reducing growth. The estradiol lanes10^{-8} and 10^{-9} signaled at roughly equivalent intensity as the control, which was not predicted. In previous examinations of the cell line by Wilson, Bobseine, Grey in a preliminary characterization of the cell line, estradiol was observed to increase growth, implying that the PCNA blot was performed using protein concentrations that exceeded the sensitive range of the blot (Wilson, 2004). The 10^{-12} lane of estradiol showed reduced signaling, similar to the Promensil lanes, indicating that the lower concentration of estradiol grew slower than the control, which is does not correspond with the predicted effects of estradiol. This result implies that the 10^{-12} lane was run using a sample with less original protein concentration, or that the control was run using elevated protein levels, which could explain why the cells exposed to 10^{-8} and 10^{-9} lanes had signaling equal to the control. The PCNA blot to measure cell growth in estradiol and Promensil deserves further examination using lower

base protein concentrations in order to further understand if T47D-KBluc is responsive to both estradiol and Promensil.

Over the multiple examinations of T47D-KBluc, no conclusion could be drawn on the cell lines ability to luminesce when exposed to luciferin. Using multiple settings and measuring techniques, T47D-KBluc no specific luminescence was detected. The nonspecific emission spectrum suggested that either the assay itself was not working or that the instrumentation settings were not optimized for detection. A florescence spectrophotometer, which is designed to record light emitted from samples in an excited state, was used to record the luminescence of the experiments. This machine should have been able to record emitted light in the absence of excitation, but it is not the ideal machine for the application. The sensitivity of the florescence spectrophotometer could not be identified due to the lack of a positive control, a solution that was known to emit light at a similar wavelength to the luciferase reaction. A luminometer is designed to record light without using a light source to excite the sample and measure only the light produced by a reaction. Without checking the luciferase reaction in both machines with a positive control, it cannot be determined if T47D-KBluc produces luciferase when the estrogen receptor is activated.

The lack of results from the luciferase assay could indicate that the particular cells being used may have mutated since the original T47D-kBluc cell line was received and cultured. Because the cultures underwent numerous splitting stages and growth on multiple types of media, cells could have been accidently selected for a strain of T47 that no longer had a functioning luciferase reporter or an unknown error in the pathway that no longer allows for luciferase be transcribed.

Ultimately, our assays were unable to distinctly endorse the T47D-KBluc as a model candidate for further investigation of phytoestrogens. However, the cell line requires further examination using both of the previously used techniques, PCNA and luciferase assays. The PCNA blot did indicate that the cell line reacted as expected to the Promensil and estradiol. This blot should be repeated using a reduced

protein density, in order to get a more appropriate positive control from cells dosed with estradiol. A positive control from cells exposed to estradiol would strength the results seen in the study.

For the luciferase assay, a preliminary step can be made. To determine if the cell line we possess can still accurately report using the assay, rtPCR, a gene sequencing technique, can be used to determine if the cells still contain the correct genes required for the transcription of luciferase. This examination would only be necessary if the use of a luminometer with a positive control have been ruled out as viable examination techniques.

This examination of T47D-KBluc determined that the cell line has potential for being a model cell line to examine Promensil and other phytoestrogen drugs. If the luciferase assay is determined to respond to estradiol and Promensil in a measureable way, the cell line can be used to test individual components that make up the drug. Promesil is not made up of pure known substances, but a cocktail of components that are contained in red clover. High performance liquid chromatography, or HPLC, has revealed a multitude of different phytoestrogens including but not limited to Daidzein and Formononetin (Setchell, 2001). The extract used in the study has an effect on the cell line, but it is unclear as to which components of Promensil can induce changes. To further examine Promesil with a functioning luciferase assay, the individual phytoestrogens identified could be purified and applied to the cells to determine which chemicals are acting on the estrogen receptor. PCNA could also be used to understand which purified phytoestrogens reduce growth and if any increase growth, which could prove that Promensil is not an ideal drug composition and deserves purification. With each component of Promensil being analyzed for its effect on growth and for its action in the estrogen pathway, a drug with more accurately calculated ingredients to counteract menopausal symptoms while having anti-carcinogenic affect could be developed.

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